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Research Article

Inter-laboratory ring test for environmental DNA extraction protocols: implications for marine megafauna detection using three novel qPCR assays

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Abstract

The comparability of methods applied to environmental DNA (eDNA) samples across laboratories remains a significant challenge for biodiversity monitoring on a global scale. Performance differences between protocols can jeopardize effective conservation strategies across regions and focal species. To address potential discrepancies amongst four international partners within a collaborative eDNA initiative, an inter-laboratory comparison (i.e., ring test) was conducted to compare efficiencies of established DNA extraction methodologies based on 39 eDNA samples. Each laboratory contributed eight to eleven samples collected throughout the North-East Atlantic and the Mediterranean Sea near sperm whales, porbeagle sharks, basking sharks, bottlenose dolphins and common dolphins. After lysis, aliquots were exchanged between laboratories before subsequent DNA extraction using each facility's preferred method. Extracts were returned to the lysates' respective laboratories of origin for measurements of total DNA concentration, as well as quantitative PCR using three novel species-specific assays for marine megafauna. Our findings revealed similar concentrations of total DNA, yet a significant reduction in extraction performance for targeted qPCR reactions by one laboratory, who has therefore modified their extraction method to be used for the remainder of this project. Overall, detection success differed based on the target taxa with sharks being less often detected (and at lower concentrations) than marine mammals. Significant interaction effects were found between combinations of laboratories and species, suggesting a link between extraction protocols and variable environmental conditions. Our study serves as a foundational step towards establishing reproducible practices that are crucial for the success of multinational eDNA projects to enable comparable results.

Key words: Cetaceans, *Cetorhinus maximus*, *Lamna nasus*, Limit of Detection, optimization, *Physeter macrocephalus*, ring test, targeted assay, *Tursiops truncatus*

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Introduction

Advances in molecular technologies have revolutionized the collective perception and capabilities of assessing biodiversity. Over the past fifteen years, environmental DNA (eDNA) has burgeoned as a noteworthy tool for monitoring the diversity of a system (Beng and Corlett 2020; Rourke et al. 2022). Through the collection, extraction and analysis of trace amounts of genetic material shed by organisms into their environment, researchers can now detect the presence of species in environmental samples such as sediment, water, snow or air (Ficetola et al. 2008; Lynggaard et al. 2022; Miya 2022). Notably, the inherent attributes of eDNA-based approaches make them particularly suitable for the investigation of rare and/or protected species, such as marine megafauna (e.g. dolphins, whales, sharks), as their non-invasive nature eliminates the need for direct contact with the animals (Foote et al. 2012; Juhel et al. 2021; Rojahn et al. 2021). Although eDNA methods are increasingly used by ecologists, especially for such studies on elusive species, their integration into large-scale routine management and decision-making processes remains limited. A prominent obstacle is the need for rigorous international standards and optimized protocols, which could make eDNA-based monitoring more reliable and comparable across initiatives.

Considering the highly sensitive nature of methodological choices, efforts to standardize sampling and analysis methods are crucial, especially within the framework of large international projects. The multifarious nature of biological systems, coupled with the rapid evolution of technology, presents significant challenges to standardization efforts (Thomsen and Willerslev 2015; Goldberg et al. 2016; Bruce et al. 2021; Buxton et al. 2021; Thalinger et al. 2021). Variability in the techniques for sample collection and processing can lead to discrepancies in data interpretation and conclusions, undermining the reproducibility of research findings (Katano et al. 2017; Bruce et al. 2021; Buxton et al. 2021). For instance, the volume of water collected directly affects the quantity of target-specific eDNA in a sample, with larger volumes (> 5 L) generally yielding higher amounts of target DNA (Calderón-Sanou et al. 2020; Zhang et al. 2023). This creates challenges in projects aimed at monitoring marine megafauna, in which detecting taxa is difficult due to low concentrations of target DNA amidst genetic material from various non-target organisms (Parsons et al. 2018; Valsecchi et al. 2020; Suarez-Bregua et al. 2022). Additionally, factors such as decontamination protocols, time from sample collection to filtration and sample storage further influence the integrity and detectability of eDNA. Recognizing these challenges, recent eDNA-based studies have shown the need for comprehensive reviews and guidelines to improve the reliability of eDNA methodologies and subsequent analyses (Rourke et al. 2022).

For international projects, variations in technical expertise, resources (e.g. field or laboratory equipment) and regulatory environments across participating laboratories can exacerbate inter-institutional inconsistencies. These challenges have been addressed in several guidelines, which summarize best practices in eDNA research from preliminary sampling to post hoc bioinformatic processing (Loeza-Quintana et al. 2020; Minamoto et al. 2021; Morisette et al. 2021; Bruce et al. 2021; Blancher et al. 2022). Furthermore, international working groups consisting of eDNA specialists are being established to monitor and

assess current methods and applications of eDNA research. Examples include a subgroup of the European Committee for Standardization (CEN/TC 230/WG 28; https://www.cencenelec.eu/), the USA Government eDNA Working Group (GeDWG; usgs.gov) and the international eDNA Society (ednasociety.org).

eDNA-based projects are subject to the protocol used for extracting the genetic material from the environmental sample. DNA extraction encompasses a series of intricate steps, including cellular lysis, DNA isolation, protein and contaminant washing and final elution of high-quality DNA (Knebelsberger and Stöger 2012; Barbosa et al. 2016). The widespread adoption of commercial kits, with the necessary reagents for extracting DNA from a variety of mediums (e.g. tissue, water, soil), have become common practice throughout recent years as they provide easily accessible, streamlined and reproducible protocol for retrieving genetic material from a sample (Lear et al. 2018; Pearman et al. 2020). Some of the most widely used and recommended commercially available extraction kits are Qiagen's (Venlo, The Netherlands) DNeasy Kit, which is recommended by the official manual for eDNA research published by the eDNA Society (Ficetola et al. 2008; Lear et al. 2018; Tsuji et al. 2019; Minamoto et al. 2021) and the PowerWater DNA Isolation Kit (Mobio, Hilden, Germany). According to a review of eDNA extraction approaches by Kumar et al. (2020), a distinguishing feature of some kits (such as the PowerWater Kit) lies in its built-in PCR inhibitor removal step, which can also be conducted after extraction is carried out (e.g. with Zymo OneStep PCR Inhibitor Removal Kit). This is particularly relevant for environments with high levels of suspended particulate matter or poor water quality, which are likely the source of PCR inhibitors, such as humic acids, fulvic acids and polysaccharides (Kuhn et al. 2017; Lear et al. 2018). However, inhibitor removal also introduces the potential of losing target DNA due to increased agitation of the lysate (McKee et al. 2015; Goldberg et al. 2016) and the inclusion of this step does not guarantee superior extracts. Consequently, the efficacy of the chosen extraction and potential inhibitor-removal approach is contingent upon its compatibility with the specific taxonomic, geographical and environmental attributes of the study, warranting meticulous consideration.

Following extraction, total DNA concentration in an extract can be measured via spectrophotometry or fluorometry (Brunker 2020; García-Alegría et al. 2020). Meanwhile, targeted approaches, such as quantitative PCR (qPCR) and droplet digital PCR (ddPCR) can be used to ascertain the presence and abundance of a specific species within the sampled environment by discerning particular genetic traces of interest amidst a heterogeneous sample, in which the quantity of target DNA is likely present at very low concentrations (Goldberg et al. 2016; Hunter et al. 2017). qPCR methods (with assays either using an intercalating dye or a fluorescently labeled probe for quantification) are the most widely used technique for attaining species-specific detections (Thalinger et al. 2021). However, its success depends upon precise assay design entailing selectivity that precludes the amplification of non-target taxa co-existing with the focal species. Moreover, a rigorous validation regimen spanning *in silico*, *in vitro* and *in situ* evaluations is needed to forestall spurious reactions (primer dimers, hairpins etc.) and enhance the applicability to eDNA samples collected from the field. These testing protocols have recently been presented by Thalinger et al. (2021) as a 5-level validation scale, beginning at Level 1 with simple *in silico*

and non-target tissue testing to Level 5 with statistical testing of an assay's detection probability, as well as modeling with ecological and physical factors which may influence the rate of perception of a sample's DNA (Garafutdinov et al. 2020; Klymus et al. 2020).

The Limit of Detection (LOD) and Limit of Quantification (LOQ) are two metrics that describe the sensitivity and quantitative precision of DNA assays. The LOD delineates the lowest amount of DNA that can be consistently detected, while the LOQ specifies the minimum level at which DNA can not only be detected, but also quantified with acceptable accuracy and precision (Hunter et al. 2017; Klymus et al. 2020; Thalinger et al. 2021). However, caution must be exercised when accounting for these metrics during data analysis, particularly when interpreting positive amplifications which fall below an assay's Limit of Detection, as highlighted by Klymus et al. (2020). Taking a conservative approach by excluding data below LOD may result in the loss of actual detections of the target species. Conversely, the LOD can serve as a comparative benchmark for inter-laboratory processing of the same sample.

Profoundly influencing an assay's LOD and LOQ is the design of qPCR primers (and often a fluorescently labeled probe), a process which is now often supplemented by advanced machine learning or automation (Kronenberger et al. 2022; Allison et al. 2023). The careful design process ensures the sensitivity and specificity of qPCR, which is vital for distinguishing low abundance targets (Wilcox et al. 2013; Rees et al. 2014). Furthermore, the choice of PCR chemistry and cycling conditions is equally critical for optimizing assay performance (Klymus et al. 2020; Langlois et al. 2021). In summary, the analytical workflow of eDNA extraction and analysis involves intricate considerations ranging from the means of obtaining DNA from a sample (e.g. the selected extraction kit) to the development and refinement of a species-specific assay. Although this is only part of the eDNA workflow, the manifold options available for DNA extraction and target DNA amplification already make comparative tests a requirement before direct data comparisons and applications.

A ring test, for example, is a structured comparative test that is used to evaluate the reliability of certain laboratory procedures, by having all partners of a collaborative project analyze the same samples followed by a comparison of the obtained results. The application of a ring test can identify deviations between laboratories that are participating in a project and detect discrepancies in methods which affect the outcomes (Paton et al. 2000; Vasselon et al. 2021). The structure of a ring test includes steps for sample preparation and distribution, analysis and comparative reporting, in which the outcome highlights the performance of each laboratory including any significant deviations from the expected results. Though a ring test can identify variability and ensure consistency, there are drawbacks stemming from basic differences in sample handling, environmental conditions (e.g. one laboratory may have different air flow or humidity) and instrument calibration, which can produce spurious results and lead to false conclusions. Therefore, ring tests are a powerful tool for comparing the performance of different partners in a project, but require careful consideration of the obtained results before adjusting underperforming processing steps.

In the context of international efforts to enhance the detection rates of marine megafauna DNA from environmental samples, a key focus has consistently

been on refining both field sampling and laboratory protocols. This endeavor led to the initiation of a ring test involving four laboratories, collaboratively working in an international research project entitled eWHALE and aiming to study marine megafauna across the North-East Atlantic and Mediterranean Sea using eDNA-based methods. The four laboratories are: University of Innsbruck (UIBK; Austria), the National Research Institute for Agriculture, Food, and the Environment (INRAE; France), University College Cork (UCC; Ireland) and the Institute for Marine Research (IMR; Norway), each relying upon specialized molecular techniques, particularly eDNA extraction methods. Our aim was to compare the efficiency of extraction protocols for a variety of eDNA samples collected around various marine megafauna species. Additionally, three species-specific qPCR assays, which can be utilized by other eDNA specialists in future studies, were developed to specifically amplify sperm whale (*Physeter macrocephalus*), porbeagle shark (*Lamna nasus*) and basking shark (*Cetorhinus maximus*) DNA from environmental samples. We aimed to evaluate the efficacy of laboratory-specific extraction techniques by comparing both total DNA yield and target species DNA yield. This evaluation is crucial for improving the standardization of eDNA monitoring methodologies across various institutions for the purpose of assessing mobile species with spatial ranges beyond country borders.

Methods

Field sampling

In summer 2023, water samples were collected from different regions throughout the North-East Atlantic and Mediterranean Sea by researchers, students and partners who were trained in eDNA sampling (Fig. 1; Suppl. material 1). Prior to the ship leaving the harbor, buckets and tubing were rinsed with household bleach diluted 1:10, rinsed thoroughly with tap water, then left to dry. The entire cleaning procedure was carried out wearing DNA-free gloves. At sea, samples were filtered from the surface of the water column through different commercially available environmental DNA filters: Smith-Root (Vancouver, USA), Sylphium (Sylphium molecular ecology, Groningen, The Netherlands) and Sterivex™ (Millipore®; Merck Chemicals and Life Science GesmbH, Darmstadt, Germany; Table 1). At the end of filtration, all filters were dried by running the pump for an additional 30 s to 1 min outside of the water or pushing air through the filter with a syringe. Storage buffer consisting of TES buffer (0.1 M TRIS, 10 mM EDTA, 2% sodium dodecyl sulfate; pH 8) and proteinase K (20 mg/mL) in a ratio of 190:1 was added to each filter (1.5–3 mL depending on the filter type, see below), except for Smith-Root filters. Between sampling events, the tubing and buckets were rinsed three times with seawater to prevent direct cross-contamination. All participating eWHALE partners were involved in a large-scale eDNA field sampling optimization initiative during the summer 2023. The present extraction ring test was carried out with a subset of these samples. Therefore, sample volumes and eDNA filters vary considerably amongst partners and sampling locations. Below, we describe the sampling regimes that each partner followed for their subset of samples that were selected to be used in this ring test.

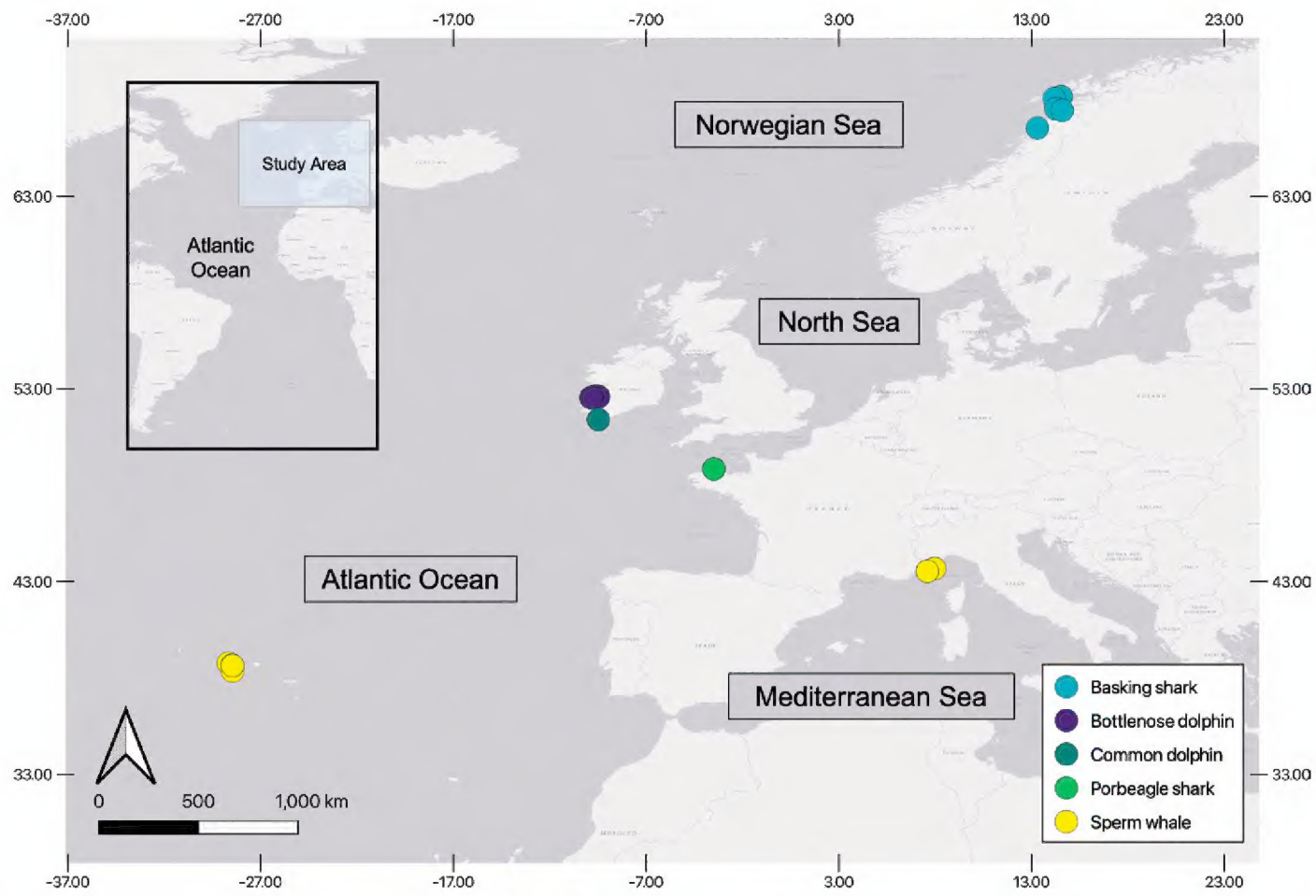

Figure 1. Locations in which eDNA samples analyzed for this ring test were collected. Points are colored according to the target species. Cartography was created using QGIS v. 3.34.3 using ESRI basemap services (Esri, DeLorme, HERE, MapmyIndia).

Table 1. Overview of the eDNA sampling procedures, target species and extraction methods per participating laboratory.

Parameter	UIBK	INRAE	UCC	IMR
Filtration technique	Peristaltic pump	Suction pump	Syringes	Peristaltic pump
eDNA filter	Smith-Root (n = 6), Sylphium (n = 5)	Sylphium (n = 10)	Sterivex (n = 10)	Sterivex (n = 8)
Filter pore size (µm)	1.2; 0.8	0.8	0.45	0.45
Filter material	Polyethersulfone (PES)	Polyethersulfone (PES)	Polyvinylidene fluoride (PVDF)	Polyvinylidene fluoride (PVDF)
Volume of water filtered (L)	10	5.75	1.5–2	5
Target species	Sperm whale (*Physeter macrocephalus*)	Porbeagle shark (*Lamna nasus*)	Bottlenose dolphin (*Tursiops truncatus*) and Common dolphin (*Delphinus delphis*)	Basking shark (*Cetorhinus maximus*)
Extraction method	Qiagen BioSprint® 96 Workstation using the Biosprint 96 tissue protocol **	Macherey-Nagel NucleoSpin Tissue Kit**	Qiagen DNeasy Blood and Tissue Kit**	Qiagen DNeasy Blood and Tissue Kit**
Modification to the lysis and extraction protocol compared to manufacturers' instructions	100 µL of TE buffer instead of AE buffer for elution	Addition of 25 µL proteinase K at the lysis step; Buffer BE was heated at 70 °C and elution was repeated twice with **the same 100 µL** of Buffer BE with 3-minute incubation time	Lysates were incubated at 56 °C for 1 hr prior to extraction	A QiaVAC 24 Plus vacuum system (ID: 19413, QIAGEN) was used instead of centrifugation for spin column steps
Link to detailed extraction protocol	http://dx.doi.org/10.17504/protocols.io.q26g71p83gwz/v1	http://dx.doi.org/10.17504/protocols.io.rm7vzxexrgx1/v1	http://dx.doi.org/10.17504/protocols.io.n92ld8m2ov5b/v1	http://dx.doi.org/10.17504/protocols.io.n92ld8m2ov5b/v1
Sanger sequencing	Eurofins Genomics Germany GmbH (Ebersberg, Germany)	GenoScreen (Lille, France)	Eurofins Genomics Germany GmbH (Ebersberg, Germany)	University Hospital of North Norway (Tromsø, Norway)

** Modifications made to protocol (see main text for details).

In the Mediterranean Sea, samples were collected in volumes of either 2, 5 or 10 L with a bucket. Out of 68 total samples collected throughout the season, 17 samples were collected in close proximity to sperm whales (*Physeter macrocephalus*; Fig. 1). Water samples were filtered through self-preserving Smith-Root filter

capsules (1.2 µm filter pore size) using a peristaltic pump (Solinst; Model 410; Thomas et al. (2019)). Filter capsules were stored at 4 °C on board then in a facility in the harbor of San Remo (Italy) following the cruise until a subset (n = 6 sperm whale samples) was shipped to UIBK in October 2023 for subsequent analysis.

In the North-Eastern Atlantic Ocean waters around the Azores islands of Faial and Pico (Fig. 1), researchers aboard CW Azores whale watching cruises (cwazores.com) used a bucket to collect 10 L of water (n = 42 samples) from sperm whale flukeprints, which were filtered through Sylphium filter capsules (0.8 µm filter pore size; ID: SYL010-08-20) using a peristaltic pump (ID: 12.34. SB; Eijkelkamp, Giesbeek, The Netherlands). All filters were filled with 1.5 mL of storage buffer, which included an Internal Positive Control (IPC), an artificial fragment of DNA used for quality control, from Sinsoma GmbH (https://www.sinsoma.com/en/). eDNA filters were stored at the University of the Azores in a -20 °C freezer until being transported to UIBK in July 2023 for subsequent analysis (n = 5 used in this study).

In the French National Nature Reserve of the Seven Islands in Brittany (Fig. 1), a total of 10 water samples targeting porbeagle sharks (*Lamna nasus*) eDNA were collected between June and September 2023. Five water samples were collected in 5.75 L containers and fully filtered through Sylphium capsules (0.8 µm filter pore size; ID: SYL010-08-20) using a suction pump. The other five water samples were directly filtered from the water using the same type of capsules and the same pump for 5 minutes. Once the filters were pumped dry, 3 mL of storage buffer were added. The filters were then stored at -20 °C until analysis at INRAE (n = 10 used in this study).

In the Shannon Estuary, nine water samples were collected with a 12 L bucket from the fluke prints of bottlenose dolphins (*Tursiops truncatus*). One short-beaked common dolphin (*Delphinus delphis*) sample was collected in the same manner off the South-West Coast of Ireland (near Baltimore, Cape Clear Island; Fig. 1). From these water samples, between 1.5 and 2 L were filtered through Sterivex-HV filter capsules (0.45 µm pore size; Merck Millipore ID: SVHV010RS) using 50 mL disposable syringes. Afterwards, 1.5 mL of storage buffer were added. The filter capsules were stored in a cooler on ice before being transferred to a -20 °C freezer upon return to the laboratories at UCC's North Mall Campus (n = 10 used in this study).

In the Norwegian Sea by the Lofoten Islands (Fig. 1), eight surface water samples were collected targeting basking sharks (*Cetorhinus maximus*). Each 5 L sample was filtered through Sterivex-HV filter capsules (0.45 µm pore size) using a peristaltic pump. A 50 mL syringe was used to push air through the filters before 1.5 mL storage buffer was added. Filters were stored at -20 °C until further analysis at IMR (n = 8 used in this study).

Sample lysis and extraction

All filters were incubated for 3 h at 56 °C. Prior to incubation, each Smith-Root filter was removed from its housing (using DNA-free forceps) then soaked with 400 µL of storage buffer. After incubation, each Smith-Root filter was transferred into a plastic inlet placed inside the original reaction tube and centrifuged at 18,626 g for 10 min to separate the lysate from the filter. For Sylphium filter capsules, lysis buffer was removed using a 3 mL or 6 mL syringe, resulting in 1 to 1.4 mL lysate

per sample for UIBK and 2.25 to 6 mL for INRAE. Lysis buffer was removed from Sterivex filters using a 2 mL syringe, resulting in 0.5 to 2.0 mL of lysate per sample.

Each laboratory received a 250 µL aliquot of lysate per eDNA sample. Samples with less than 1 mL lysate were diluted with TES buffer to 1 mL total volume before aliquoting. At UIBK, an extraction IPC (IPC-L: approximately 5,000 copies per sample; Sinsoma GmbH) was added to each aliquot. Generally, lysates were stored at -80 °C prior to shipping (packaged with ice in Styrofoam containers) between project partners in autumn 2023. We opted for overnight shipping whenever possible, but in some cases, lysates took 1–2 days to arrive at their final destination. Once eDNA lysates arrived at their destination, they were stored at -80 °C or -20 °C prior to further analysis. For a conceptual overview regarding the processing and sample exchange steps from primary lysis, extraction and DNA quantification, please refer to Fig. 2.

Extraction protocol per laboratory

Each project partner employed a DNA extraction protocol that is commonly used in their facility for high numbers of eDNA samples; each including slight modifications from the original manufacturer's protocols (Table 1). All extractions were performed in dedicated laboratory spaces with proper ventilation and cleaning procedures adhering to the processing of eDNA samples (e.g. surface cleaning with bleach, sterilized DNA-free gloves and protective wear; Hymus (2016); Thalinger et al. (2021)). Additionally, PCR preparation was conducted in separate rooms with appropriate PCR-dedicated workbenches that are disinfected by UV light at least once per working day.

Per extract, four aliquots (25 µL each) were generated, three of which were sent back to their laboratory of origin for further analysis using the same shipping conditions as before. The final aliquot remained as a backup at the laboratory carrying out the extraction.

Total DNA quantification

Each project partner measured the total DNA and the target DNA of extracts from their original lysates (e.g. UIBK measured the extracts generated from the 11 sperm whale eDNA samples for extracts created at all participating laboratories: UIBK, INRAE, UCC and IMR; Fig. 2). Total DNA concentrations (ng/µL) per extract were measured via a Qubit™ fluorometer using the Qubit dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, Carlsbad, California, US; ID: Q32851). Qubit standards and DNA sample tubes were prepared using low-bind tubes (ID: Q32856; Thermo Fisher Scientific, Waltham, MA, USA) and 5 µL of extract (protocol: https://dx.doi.org/10.17504/protocols.io.bc6vize6). All tubes were measured in triplicate.

Species-specific eDNA quantification

Assay development and validation

Targeted qPCR TaqMan MGB assays were developed for this study in order to amplify DNA from the species of interest for field samples contributed by UIBK, INRAE and IMR. Primarily, full mitochondrial sequences from target and

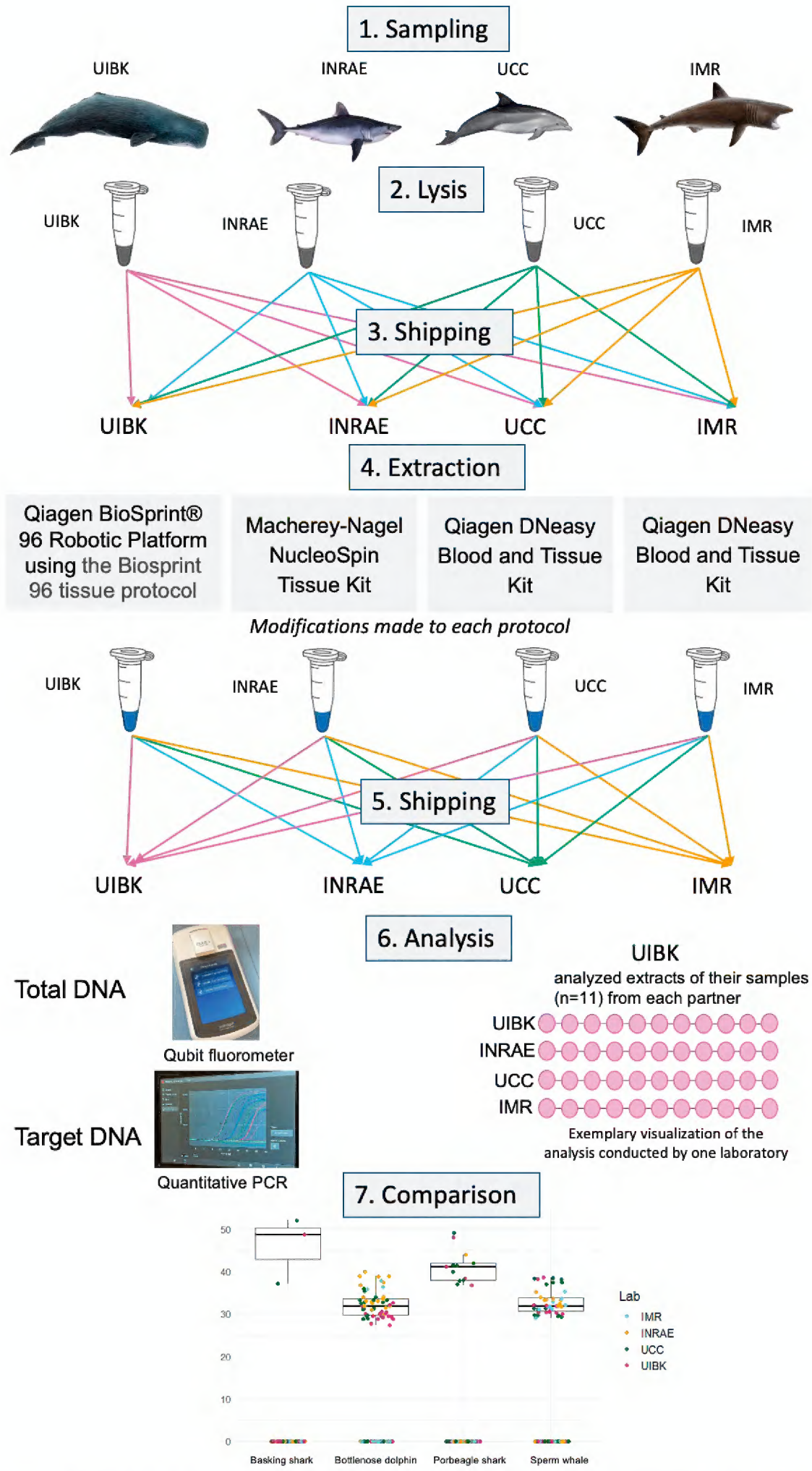

Figure 2. Conceptual diagram summarizing the workflow of lysate and DNA extracts by the four laboratories involved in the ring test. Species icon attributions: Sperm whale (*Physeter* macrocephalus) image from pngimg.com, https://pngimg.com/image/109659, Porbeagle shark (*Lamna* nasus) image from an online article available at https://untamedscience.com/biodiversity/porbeagle-shark/ written by Lindsay VanSomeren, Bottlenose dolphin (*Tursiops truncatus*) image from National Oceanic and Atmospheric Administration, NOAA Fisheries, www.fisheries.noaa.gov/species/common-bottlenose-dolphin, Basking shark (*Cetorhinus maximus*) image from Elding: The Whale Watching Pioneers at https://elding.is/basking-shark-cetorhinus-maximus.

non-target species (i.e. closely-related and/or co-occurring species) were obtained from publicly available repositories (GenBank database at the National Center for Biotechnology Information (NCBI), https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned with Clustal Omega (Sievers et al. 2011) and preliminary species-specific qPCR assays were selected using assayID, a

publicly available software tool (https://github.com/jammc313/assayID). This program scans the input mitochondrial sequence alignment file using Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012) to design primer/probe sets for previously defined windows across the full mitogenome. Given a DNA sequence template, Primer3 generates primer/probe sets optimized for various parameters that are critical to assay performance. This includes primer/probe length, melting temperatures (Tm), GC content and avoidance of secondary structure formations, amongst others. The software is designed to maximize specificity and efficiency in amplification, minimizing potential issues, such as dimerization or hairpin formation that can impair the qPCR assay's accuracy and sensitivity. Sequence diversity and distance metrics are calculated for the regions covered by the designed assays, including measures of target species genetic diversity and distance measures between target and non-target sequences (e.g. Shannon Entropy, sequence similarity, nucleotide divergence). The assays are then ranked according to their specificity and sensitivity. An ideal assay will target a region that has a combination of a low genetic diversity for the target species sequences and high genetic distance to non-target species sequences. A multivariate statistical method: Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) is finally used to rank designed assays and identify those with optimal specificity and sensitivity. Assays with the highest rankings from this program were selected for further manual development and *in silico* testing (described below per lab).

Three unique species-specific TaqMan assays were ultimately optimized for the species of interest from UIBK. INRAE and IMR (Table 2; Suppl. material 2). At UIBK, a sperm whale qPCR assay was designed targeting the Cytochrome B (CYTB) region of the mitochondrial genome. Binding regions and primer lengths were manually adapted to enhance specificity (i.e. sufficient mismatches with non-target taxa), adhering to standard recommendations for TaqMan assays (Applied Biosystems Primer Express v.3.0.1; Life Technologies, Foster City, CA, USA) and minimizing the occurrence of secondary structures using BioEdit v.7 (Hall 1999, Primer3 (Untergasser et al. 2012), Primer Premiere (PREMIER Biosoft) and Primer Express 3.0.1 (Applied Biosystems). The probe was labeled with 6-FAM and MGB-Q530 quencher (5' and 3,' respectively, Table 2).

At INRAE, the assayID program identified a total of 295 primer/probe combinations for porbeagle sharks. A total of 25 combinations that met the criteria of a window size of 150–180 bp, no hairpin, oligo not ending with G and no "GGGG" string in the oligos were retained. They were BLASTed (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check for specificity with porbeagle sequences and results matching with other species or with mismatches with porbeagle sequences were excluded. The best candidate targeted the ND1 and, to improve its specificity, the last bp was manually removed from the probe. The probe was labeled with 6-FAM and a BHQ-1 quencher (5' and 3', respectively; Table 2).

At UCC, eight primer pairs were selected, three from the above-mentioned assayID program, two created using IDT PrimerQuest Tool and three from existing literature (Stoeckle et al. 2018; Greiner-Ferris 2020). The specificity and efficiency of these primers was initially tested *in vitro* (via conventional PCR and gel electrophoresis) using DNA extracts of target and non-target species (described further below). The primers designed by Greiner-Ferris (2020), targeting the displacement loop (D-loop) region of the mitochondria, were selected

Table 2. Assays used in the current study for the amplification of target species DNA.

Laboratory	Target Species	Gene	Primer/Probe Name	Forward (5'-3') Reverse (5'-3') MGB Probe (5'-3')	Fragment length (bp)	Optimal Annealing Temp. °C	Assay LOD**
UIBK	Sperm whale (*Physeter macrocephalus*)	CYTB	Phy-cat-S939 * Phy-cat-A939 * P030_Phy-cat *	CCTACCACACAATCAAAGACACC GGTTTGATGTGTGTTGGGGTAT TAGTGGATTTGCTGGGGTGTA	144	61	0.0001
INRAE	Porbeagle shark (*Lamna nasus*)	ND1	LnND1-F209 * LnND1-R380 * LnND1-P242 *	TCAGCATCTTCCCCTTTCCT ATCCGGAGCCCAAGATAGTG CCCACAATGGCTCTTACACTGGCCCTCCT	172	60	0.000526
UCC	Bottlenose dolphin (*Tursiops truncatus*) and Common dolphin (*Delphinus delphis*)	D-loop	TtDloopF TtDloopR	CACACGTGCATGCTAATATTTAG GAGTGACCATAGGATATAATGGAG	159	60	0.00001
IMR	Basking shark (*Cetorhinus maximus*)	ND5	CetoMaxND5F_01 * CetoMaxND5R_01 * CetoMaxND5P_01 *	AGTTTCCGCCCTACTCCATT GCTGCGGTAAAGAGGGTAGT AGTCGTTGCCGGCGTCTTCCTGCTA	144	60	0.0001

* Created for this study
** As described by the discrete method presented in Klymus et al. (2021).

because they were the most specific to the target species (Table 2; Suppl. material 2). The last base pair at the 3' end of the reverse primer was removed so that the primers would better amplify the bottlenose dolphin haplotypes found in the study area (Nykänen et al. 2019). A putative probe for TaqMan chemistry was initially designed for this modified version of the primer pair using the IDT PrimerQuest™ Tool. The probe/primer assay was then extensively tested using standard dilutions of tissue-derived bottlenose dolphin DNA, but failed to detect target DNA beyond 0.01 ng/µL. The primers were tested using SYBRgreen mastermix (without the use of a probe) in which it was possible to detect target DNA beyond 0.001 ng/µL. Thus, it was decided that all subsequent runs would be on SYBRgreen-based chemistry using the aforementioned primer pair.

At IMR, the assayID program resulted in several potential assays for basking sharks. Further *in silico* testing for target species specificity and tendency to form secondary structures using Primer-BLAST (Ye et al. 2012) and Integrated DNA Technologies OligoAnalyzer Tool (Owczarzy et al. 2008), resulted in the selection of the best performing assay targeting part of the ND5 region. The probe was labeled with 6-FAM and NFQ-MGB quencher (5' and 3,' respectively, Table 2).

The specificity for all assays presented herein was verified in silico via Primer-BLAST (Ye et al. 2012), with standard settings and the nr database. No amplification of closely-related or co-occurring species was found for UIBK, IMR and INRAE assays. The assay of UCC amplified all Delphinidae species, including the target species bottlenose dolphin and common dolphin. At UIBK, a gradient qPCR was performed to optimize the melting temperature to be used in subsequent reactions. Triplicate dilution series of sperm whale DNA (dilution steps: 1:10; starting conc. 1 ng/µL, 6 points) and one No Template Control (NTC) and nine Negative Controls were included on the plate. The optimal melting temperature for this assay was 61 °C (Table 3). At INRAE, a gradient qPCR was also performed to optimize the assay's melting temperature. Triplicate dilution series of porbeagle shark DNA (dilution steps: 1:10; starting conc. 0.526 ng/µL, 6 points), one NTC and one Negative Control were included on the plate. The optimal melting temperature for this assay was 60 °C. DNA extracts from target and non-target species were used for *in vitro* testing of all

Table 3. Description of qPCRs per participating laboratory.

qPCR parameter			UIBK	INRAE	UCC	IMR
qPCR Cycler			qTOWER3G (Jena, Germany)	BioRad CFX96 (Bio-Rad Laboratories, Hercules, CA)	Applied Biosystems 7500 Real-Time PCR System (Foster City, CA)	Applied Biosystems QuantStudio Flex 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA)
Marine Megafauna Assays		Master Mix (µL)	5 2× TaqMan Environmental Master Mix (ID: 4396838, EMM, Life Technologies)	10 2× TaqMan Environmental Master Mix	5 2× SYBR™ Green PCR Master Mix (ID: 4309155, Applied Biosystems™)	5 2× PerfeCTa qPCR Master Mix (ID: 101419-220, QuantaBio)
		Primer conc. (µM)	0.4 per primer	2 per primer	0.4 per primer	0.5 150 nM TaqMan Custom gene expression assay
		Probe conc. (µM)	0.2	1	NA	See above
		Nuclease-free water (µL)	1	4	2.2	1.3
		eDNA extract, NC, NTC (µL)	3	3	2	2
	Cycling Conditions	Activation	95 °C for 10 min	95 °C for 10 min	95 °C for 10 min	95 °C for 10 min
		Denaturation	40 cycles at 95 °C for 15 s	49 cycles at 95 °C for 30 s	40 cycles at 95 °C for 15 s	55 cycles at 95 °C for 15 s
		Annealing/Extension	40 cycles at 61 °C for 90 s	49 cycles at 60 °C for 50 s	40 cycles at 60 °C for 1 min	55 cycles at 60 °C for 1 min
Internal Positive Control	**Purpose**		IPC-F: gauge amount of DNA that is lost during transportation and storage of eDNA filtersIPC-L: amount of DNA that is lost during extraction	NA	Kavlick IPC to test for possible inhibitors or laboratory processing error	ThermoFisher IPC Exo DNA to test for possible inhibitors or laboratory processing error
	Internal Positive Control in duplex with target species assay?		No	NA	No	Yes - 1 µL of ThermoFisher IPC Exo Mix and 0.2 µL of ThermoFisher IPC Exo DNA were added to each reaction
	Chemistry	Master Mix (µL)	5 2× TaqMan Environmental Master Mix (ID: 4396838, EMM, Life Technologies)	NA	5 2× Taqman Universal PCR Master Mix (ID: 4304437, EMM, Life Technologies)	See above target species chemistry
		Primer conc. (µM)	0.5 per primer (Sinsoma GmbH)	NA	0.4 per primer	See above target species chemistry
		Probe conc. (µM)	0.4 (Sinsoma GmbH)	NA	0.4	See above target species chemistry
		Nuclease-free water (µL)	0.6	NA	2.4	See above target species chemistry
		eDNA extract, NC, NTC (µL)	3	NA	1	See above target species chemistry
	Cycling Conditions	Activation	95 °C for 10 min (activation)	NA	95 °C for 10 min	See above cycling conditions
		Denaturation	40 cycles at 95 °C for 15 s	NA	40 cycles at 95 °C for 15 s	See above cycling conditions
		Annealing/Extension	40 cycles at 60 °C for 90 s	NA	40 cycles at 60 °C for 1 min	See above cycling conditions

selected assays' specificity. Tissue samples used for *in vitro* testing of species-specific assays were dried in a fume hood and then extracted using either the Qiagen DNeasy Blood and Tissue Kit (ID: 69504, QIAGEN) or the Macherey-Nagel NucleoSpin Tissue Kit (ID: 740952.50, Düren, Germany), following the manufacturer's instructions. Tissue from the following non-target species were

used to test primer specificity at each partner lab using the corresponding assay: UIBK - Dwarf sperm whale (*Kogia sima*), Common dolphin (*Delphinus delphis*), Harbor porpoise (*Phocoena phocoena*), Fin whale (*Balaenoptera physalus*), Grey seal (*Halichoerus grypus*) at 1 ng/µL and 0.1 ng/µL; INRAE - nursehound (*Scyliorhinus stellaris*; 1 ng/µL and 0.1 ng/µL); UCC - common dolphin and harbor porpoise (5 ng/µL and 0.5 ng/µL); IMR, porbeagle shark (1 ng/µL and 0.1 ng/µL).

Upon optimization of cycling conditions (Suppl. material 2), the Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated for each assay following the definitions of Klymus et al. (2020) using measurements from triplicate standard curves (per qPCR plate) of serial dilutions of target DNA of known concentrations (ng/µL; Table 2). Ultimately, there was insufficient statistical power to calculate each assay's LOQ in accordance with the defined calculation method (Klymus et al. 2020). The SYBR green-based assay applied by UCC had an expected melting temperature of 73 °C, with melting temperatures for standard dilution series (of bottlenose and common dolphin species) being 73 °C aside from the last dilution point (in which a lower Tm was sometimes observed: 71–72 °C) and field samples ranging from 71.2 °C to 73.5 °C due to the assay amplifying multiple haplotypes of bottlenose dolphins.

qPCR

Each participating laboratory performed qPCR with triplicates of each eDNA extract and triplicate serial dilutions of known concentration (UIBK IPC-F: 6 points off tenfold dilution starting at 15 copies/µL; UIBK IPC-L: 6 points of tenfold dilution starting at 1,000 copies/µL; UCC Kavlick (2018) IPC: 9 points of tenfold dilution starting at 1400 copies/µL; IMR IPC in duplex with basking shark-specific assay; all laboratories target species: 6–8 points of tenfold dilution starting at either 1 or 0.526 ng/µL) on each plate. At least one No Template Control (NTC) and Negative Control (NC) were included on each plate. Cycling chemistry and parameters for each laboratory are listed in Table 3.

Sequencing

Following positive amplification of target species DNA using qPCR, amplifications were verified via Sanger sequencing of both the forward and reverse strands. At UIBK, positive qPCR amplifications of target DNA were purified using an enzymatic treatment kit (ExoSAP-IT® Express PCR Product Cleanup Reagent; Affymetrix-USB Corporation, Santa Clara, California, USA), then sent to Eurofins Genomics Germany GmbH (Ebersberg, Germany). At INRAE, the target fragments from eDNA products yielding positive qPCR amplifications were re-amplified using end-point PCR (Suppl. material 3) and bands corresponding to the expected size on a 2% agarose gel were extracted and purified using the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Dünel, Germany) before being sequenced by GenoScreen (Lille, France). At UCC, qPCR products yielding amplifications with melting temperatures in the range between 71.2–73.5 °C were run on a 2% agarose gel at 100 V for 1 h. Bands which corresponded to the expected length of the target species fragment (200 bp) were extracted and purified using the QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands). The products were

sent to Eurofins Genomics Germany GmbH (Ebersberg, Germany). At IMR, positive qPCR amplifications of target DNA were purified using ExoSAP-IT®, Sanger sequencing reactions were performed using BigDye™ Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems™ 4404310) and sequencing products were sent to the University Hospital of North Norway (Tromsø, Norway).

Resulting sequences were trimmed to remove low quality ends using Bioedit 7.7 (Informer Technologies), AliView 1.28 (Uppsala University), Geneious 7.1.9 (Biomatters, Auckland, New Zealand) and UGENE 50.0 (Unipro). The taxonomic classification of all DNA sequences obtained from sequencing was determined using BLAST (NCBI).

Statistical analysis

Total DNA measurements (via Qubit fluorometer) were tested for statistically significant differences between laboratories using a Generalized Linear Mixed Model (GLMM) in SPSS (v. 28; IBM Corp, Armonk, NY, USA) with the following formula:

$$Y_{ijk} = \mu + \pi_i + a_j + \pi a_{ij} + \varepsilon_{ijk} \quad (1)$$

in which Y_{ijk} is the DNA concentration (in ng/µL) of the extract for the k-th observation in the j-th laboratory from the i-th source, μ is the general mean, π_i is the random effect of the i-th source in which samples originated from (i.e. the different target species), a_j is the fixed effect of the j-th laboratory performing the eDNA extraction, πa_{ij} is the random effect of the i-th source in which sample extracts originated from the j-th laboratory and ε_{ijk} is the error term.

For qPCR amplifications, statistical tests were carried out on two datasets: one with all positive amplifications of the target species (hereafter "all-inclusive dataset") and one with a subset of positive amplifications that were at or below an assay's Limit of Detection following the definition provided in Klymus et al. (2021) in which at least 95% of PCR replicates for a given standard DNA concentration is amplified (hereafter "conservative dataset"). qPCR amplifications were tested for significant differences between detection probability (based on a binary variable for target species amplification) using Pearson's Chi-Square tests. Furthermore, Chi-Square was also applied to test for significant deviations in the detection probability by species (i.e. basking shark, dolphin species, porbeagle shark, sperm whale). The detection sensitivity of positive amplifications (defined henceforth by the Ct values, a proxy for the DNA concentration within an extract; measured in triplicate per extract) were analyzed using a Kruskal-Wallis test with a post-hoc Dunn's test to identify which extraction technique had a positive or negative effect on amplification strength (i.e. high or low Ct values). High Ct values correspond to lower concentrations of target DNA within an extract since it takes a higher cycle number (Ct) for the DNA to be detected by the cycler. Statistical significance was defined for all tests at a p-value < 0.05.

Figures were created in Microsoft PowerPoint and R (v. 4.3.1) using *ggplot2* (v. 3.4.4; Wickham et al. 2024), *dplyr* (v. 1.1.3, Wickham et al. 2023) *reshape2* (v. 1.4.4; Wickham 2020) and *viridis* (v. 0.6.5; Garnier et al. 2024). All code that was used to generate figures and statistical results can be found at https://github.com/eWHALE-DNA/Ring_Test. All data used for this publication can be found in the Suppl. materials: Qubit_Data.csv and qPCR_Data.csv.

Results

Total DNA quantification

Total DNA (ng/µL) measurements for all samples did not differ significantly amongst laboratories (GLMM $p > 0.05$; Fig. 3). The sample source (i.e. the random factor) and the sample source*laboratory (i.e. target species and laboratory performing the extraction) interaction were both found significant ($p < 0.05$), with sample source having the largest effect size (Table 4). Initially, the model also considered the effect of sample transportation as a binary variable to assess potential DNA degradation during shipping. However, this factor did not significantly influence the results and led to a higher AICc, indicating a less efficient model fit (Bolker et al. 2009).

Species-specific DNA quantification

Out of 468 qPCR reactions across all target species (not including standards, NCs or NTCs), 115 successfully amplified their respective target species DNA during qPCR (all-inclusive dataset; Fig. 4, Suppl. material 5). Out of those detections, 79 exhibited DNA concentrations at or below the LOD (conservative dataset; Fig. 4; Suppl. material 5).

Across all 39 water samples, 28 yielded at least one positive qPCR reaction (out of triplicate measurements) for the associated target species in the all-inclusive dataset. For UIBK samples, the target species (sperm whale) was detected in 7 out of 11 samples: four samples with one PCR replicate from UIBK and UCC extracts and three samples with at least two PCR replicates from UIBK, INRAE, UCC and/or IMR extracts. In total, 43 out of 132 reactions (11 samples, 4 extracts each, measured in triplicate qPCRs) amplified sperm whale DNA. For INRAE samples, the target species (porbeagle shark) was detected in 8 out of 10 samples: four samples with one PCR replicate from UIBK, INRAE, and UCC extracts and four samples with at least two replicates from UIBK and UCC extracts. In total, 13 out of 120 reactions (10 samples, 4 extracts each, measured in triplicate qPCRs) amplified porbeagle shark DNA. For UCC samples, the target species (dolphin species) was detected in all 10 samples: three with one PCR replicate from INRAE, UCC and IMR extracts and seven with at least two PCR replicates from UIBK, INRAE and/or UCC extracts. In total, 56 out of 120 reactions (10 samples, 4 extracts each, measured in triplicate qPCRs) amplified dolphin DNA. Detections from UCC were confirmed with the melting curve, which was as expected for dolphin species, based on the standards. For IMR, the target species (basking shark) was detected in 3 out of 8 samples: all detections were only from one PCR replicate from either UIBK or UCC extracts. In total, 3 out of 96 reactions (8 samples, 4 extracts each, measured in triplicate qPCRs) amplified basking shark DNA. There were no amplifications of NC or NTC.

Chi-Square Tests of detection probability between extraction methods for all qPCR reactions indicated that IMR detected target species significantly less than other laboratories for both datasets (all-inclusive dataset: Chi-Square = 21.155, df = 3, $p < 0.05$, Fig. 5A; conservative dataset: Chi-Square = 14.302, df = 3, $p < 0.05$, Fig. 5B). Detection probability did not differ significantly amongst UIBK, INRAE and UCC (Fig. 5; see Suppl. material 5 for further details).

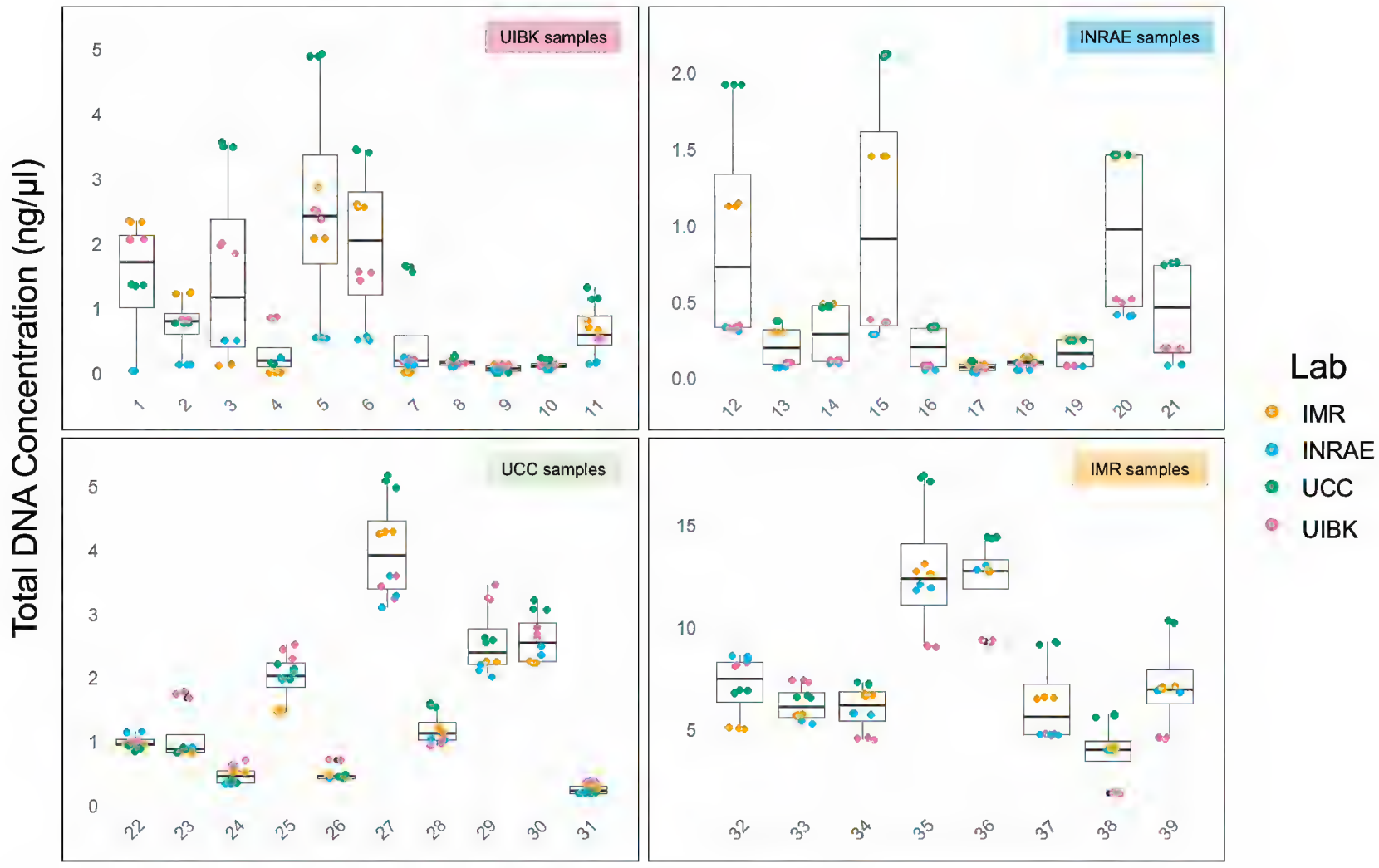

Figure 3. Total DNA concentration per eDNA sample (x-axis) and extract (ng/µL) measured in triplicate with a Qubit fluorometer. Individual points represent replicate measurements (n = 3) per extract. Note the variation of y-axis range per plot. For an expanded version of this graph, showing the exact measurements in triplicate per laboratory per sample as their own box plots, see Suppl. material 6.

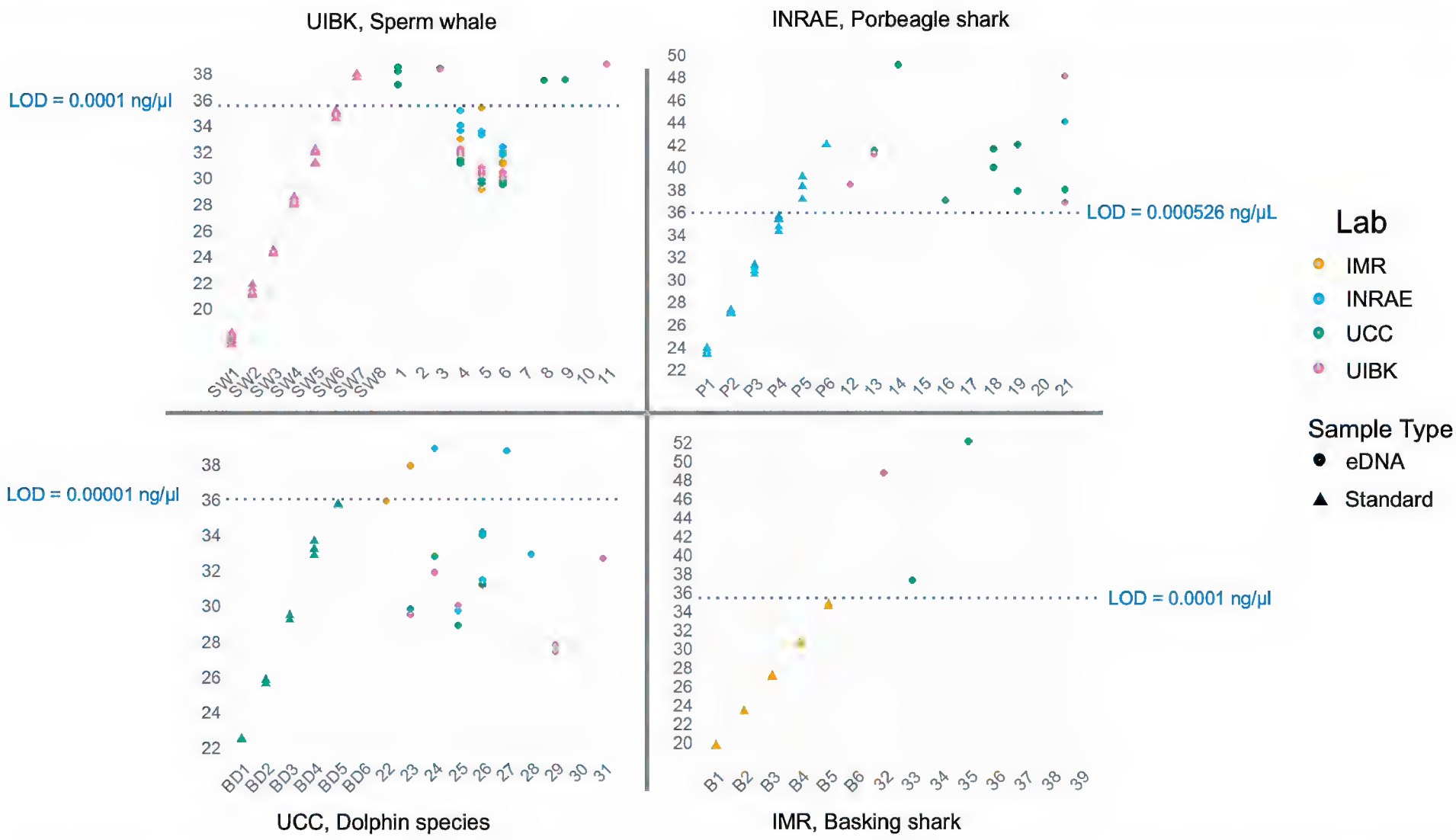

Figure 4. Species-specific detections with standard (i.e. target extract) dilution series of known DNA concentrations (ng/µL) for each lab. The LOD for each assay is denoted as a dashed blue line. Only positive detections below or at the assay's LOD (i.e. below the blue line) were included in the conservative dataset.

Across all positive amplifications, extracts generated by INRAE had significantly lower DNA concentrations (i.e. higher Ct values) than the other partner laboratories for both datasets (all-inclusive dataset: Chi-Square = 12.215, df = 3, $p < 0.05$; conservative dataset: Chi-Square = 24.322, df = 3, $p < 0.05$; Figs 4, 5). Pairwise comparisons of the extracts (Table 5) generated in different laboratories via Dunn's Multiple Comparison Test revealed a significant difference in DNA concentrations (Ct values) between UCC and UIBK for the all-inclusive dataset (Z-score = 2.730, $p < 0.05$), likely due to the variation in detections of porbeagle shark above the assay's LOD, resulting in UCC having higher Ct values on average than UIBK (Fig. 5A). Additionally, Dunn's Multiple Comparison Test identified a significant difference between the pairwise Ct values of UIBK and INRAE for both datasets (Table 5), with UIBK having an average Ct value 3.189 lower than INRAE (i.e. higher target DNA concentration) for the 10 samples in which both laboratories had positive amplifications (based on the all-inclusive dataset).

Table 4. Results from GLMM regarding the effects of extraction protocol on samples from each partner. Laboratory × Sample source indicates the interaction term between laboratory-specific extraction protocol and the source from which samples were sent from (i.e. different target species).

Model Variable		Sum of Squares	DF	Mean Square	F	Sig.
Intercept	**Hypothesis**	3742.60	1	3742.60	2.38	0.220
	Error	4712.87	3	1570.94		
Laboratory	**Hypothesis**	128.43	3	42.81	2.03	0.180
	Error	190.18	9.01	21.10		
Sample Source	**Hypothesis**	4733.47	3	1577.82	74.50	< 0.05*
	Error	190.65	9	21.18		
Laboratory × Sample Source	**Hypothesis**	190.70	9	21.19	7.24	< 0.05*
	Error	1453.83	497	2.93		

* Indicates statistical significance.
** Sum of Squares indicates the total variation in the data accounting for the presence of other factors included in the model. Degrees of Freedom (DF) indicates the number of observations of this parameter minus the number of estimated parameters. The Mean Square is the average variation explained by random effects. F is a statistic taking into account the mean square of the effect and the mean square of residuals. Significance value estimates the statistical significance of model parameters to the overall fit of the model.

Table 5. Pairwise comparisons of triplicate Ct values by laboratory-specific extraction method. Columns 2–3 represent the all-inclusive dataset with all 115 detections, whereas columns 4–5 represent the conservative dataset with only the 79 detections that were at or below the respective assay's LOD.

Institute-Institute	All-inclusive dataset		Conservative dataset (Detections at or below LOD)	
Sample 1-Sample 2	**Z-score**	**Adjusted P-value**	**Z-score**	**Adjusted P-value**
UCC-UIBK	2.730	0.019*	1.796	0.217
UCC-IMR	-0.457	1.000	0.267	1.00
UCC-INRAE	0.801	1.000	3.092	0.006*
UIBK-IMR	1.439	0.451	1.458	0.434
UIBK-INRAE	3.250	0.003*	4.907	2.77e-06*
IMR-INRAE	-1.004	0.946	-1.854	0.191

Significance values have been adjusted by the Bonferroni correction for multiple tests.
* Indicates significantly different Ct values per sample between extraction methods.

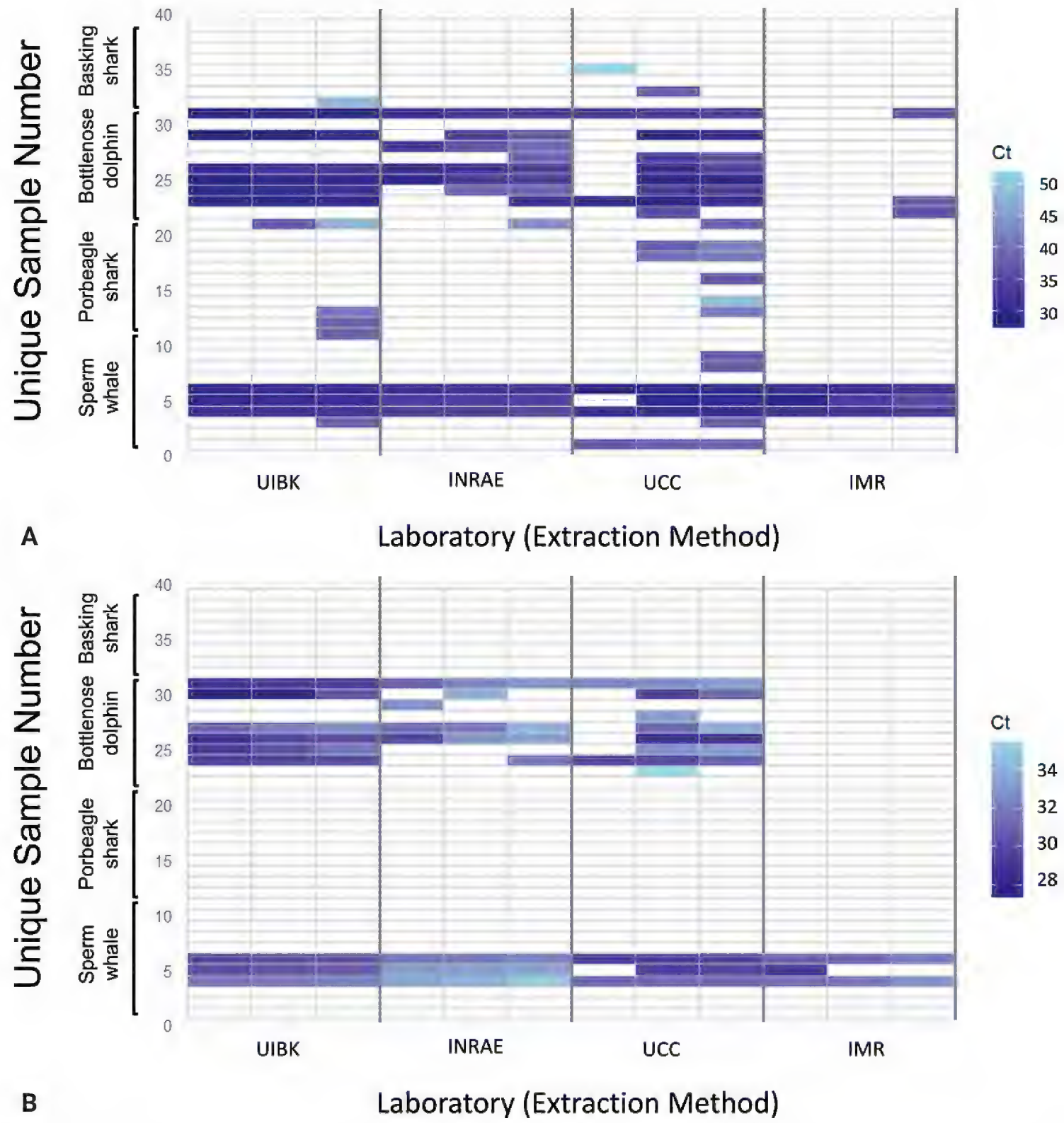

Figure 5. Results of qPCRs organized by the laboratory performing the extraction (triplicate reactions; x-axis) and the eDNA sample number and target species (y-axis). Tile color represents the Ct value; reactions without amplification were left blank. Panel **A** shows the all-inclusive dataset: all 115 detections, whereas panel **B** shows the conservative dataset: 79 positive detections that were at or below the respective assay's LOD. For alternative visualization, see Suppl. material 7.

At UIBK, nine (triplicates from 3 samples) out of 144 reactions (11 water samples extracted by four partners and analyzed in triplicate in PCR) did not detect the extraction IPC (IPC-L; though two samples were positive for the target species) suggesting either inhibition in these samples or human error (e.g. from pipetting errors). Therefore, these extracts were further assessed for inhibition by spiking 0.5 µL into a species-specific qPCR targeting *Ichthyosaura alpestris*. All amplifications of the spiked reactions showed detections with similar Ct values, indicating a lack of inhibition and pointing towards the accidental omission of IPC-L. At UCC, five out of 80 reactions (10 water samples extracted by four partners and analyzed in duplicate in PCR) did not detect the IPC – three of these reactions detected dolphins and two reactions did not detect dolphin DNA, again pointing towards an error in laboratory processing of the IPC. At IMR, 132 out of 138 qPCR reactions amplified the IPC added to the PCR master mix, the remaining six reactions contained an IPC Block (Non-Amplification Controls) and did not amplify.

Sanger Sequencing

For all positive PCR products sent for sequencing by UIBK, at least one qPCR replicate (per DNA extract) was successfully Sanger sequenced and matched to the target species (*Physeter macrocephalus*) using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). There were two instances in which the sequence was also > 97% identical to another species (Table 6). From the extracts sequenced after qPCR by INRAE, two sequences were matched to the target species (*Lamna nasus*) using BLAST. At UCC, out of the 26 PCR products sent for sequencing 13 resulted in low-quality reads (less than 50 bp) and the remaining 13 matched (> 98% identity) to the target species (*Tursiops truncatus*) using BLAST. All PCR products with a visible band were successfully matched to the target species, whereas PCR products that had no visible band on agarose gels yielded low-quality reads which could not be positively matched with any species. Of the 4 qPCR products sent for Sanger sequencing from IMR, all returned low-quality reads with no significant hits in BLAST. S Sequence data from this study, which matched the target species, are published on Figshare (Rodriguez et al. 2024; https://doi.org/10.6084/m9.figshare.27145818.v1).

Table 6. Detections of target species DNA via Sanger sequencing of positive PCR products.

Detections	UIBK	INRAE	UCC	IMR
Number of PCR products sent in for Sanger Sequencing	48	7	26	4
Number of PCR products matching to target species (> 97%)*	32	2	13	0
Number of PCR products unable to be matched to any species**	15	1	13	4
Number of PCR products matching to a non-target species (> 97%)	2 (*Drosophila* spp., *Scotophilus heathii*)	NA	NA	NA

*Available on Figshare.
**Due to sequencing error or insufficient quantity of DNA for sequencing.

Discussion

We aimed to evaluate the efficacy of laboratory-specific extraction techniques by comparing both total DNA yield and target species DNA yield from extracts generated from the same lysate by different laboratories. Our findings confirm that variations in DNA extraction methodologies significantly influenced the detection of targeted marine megafauna species in eDNA samples. Despite the general uniformity of total DNA concentrations of extracts generated from the same eDNA sample, this ring test identified significant disparities in the detections of target-specific DNA in extracts generated by IMR using their modified Qiagen DNeasy Blood and Tissue Kit extraction protocol. Three novel species-specific TaqMan assays were designed to amplify marine megafauna species. Out of all positive qPCR replicates utilizing these assays (which were sent in for Sanger sequencing), 55% were successfully sequenced with at least one positive verification for all extracts in which target DNA was detected. Notable discrepancies in detections were observed between combinations of laboratory-specific extraction protocols and target species, suggesting that the effectiveness of a particular extraction technique was highly dependent on the sample type (e.g. the type of filter used). This study exemplifies the importance of assessing gaps in the reliability of eDNA analysis protocols post field collection across multiple laboratories.

Lysates extracted by IMR with the modified Qiagen DNeasy Blood and Tissue kit yielded significantly lower detection rates for all target species for both datasets (all-inclusive and conservative regarding the assay's LOD), albeit extraction protocols being almost identical between IMR and UCC, whose DNA extracts in total had the highest number of positives in PCR (41 out of 115 reactions vs. IMR 12 out of 115 reactions). Upon reflection, two differences in the DNeasy protocol were found between IMR and UCC. The first occurred prior to extraction in which lysates were incubated for an additional hour before any subsequent work at UCC. This warming potentially improved the binding of DNA to silica membranes (i.e. reduced the chance of clogging) by preventing the precipitation of AL buffer (Lear et al. 2018). The other difference arose from the use of a vacuum at IMR during the DNA purification step, which facilitated the passage of the sample and binding buffer through the spin column. This method allowed the DNA to bind to the membrane, while other cellular components were effectively washed away. The vacuum technique versus the commonly used centrifugation protocol (which is also employed by INRAE's NucleoSpin protocol) may vary in their extraction performance due to several factors: incomplete binding of DNA resulting from insufficient vacuum pressure, inefficient washing of contaminants by the vacuum and/or higher saturation of the extraction column resulting in lower DNA yields and challenges with removing residual ethanol from the extract (Lee et al. 2018; Prasad et al. 2020). The difference between IMR and UCC's detection probability of target DNA could not be directly attributed to any of these factors, but this finding demonstrates the effect of protocol modifications for downstream analyses. Concerning differences induced by the mechanisms used for DNA binding and separation of lysate components, the extraction robot used at UIBK has been shown to be more robust in attaining the amount of total DNA within samples as the use of paramagnetic beads avoids these steps altogether (Wallinger et al. 2017). Accordingly, UIBK extracts detected the target species across multiple replicates with higher concentrations of target DNA than other laboratories (i.e. lower Ct values; average Ct across all positive amplifications: UIBK = 32.54, INRAE = 34.35, UCC = 34.82, IMR = 33.21).

All qPCR assays provided herein met Level 3 of the validation scale presented by Thalinger et al. (2021), in which the target organism was successfully detected from an environmental sample (Suppl. material 4). In all cases, the specifics of DNA extraction and concentration of eDNA from the environmental sample were reported. The assays almost satisfied Level 4 of the validation scale in which the LOD has to be calculated and *in vitro* qPCRs on co-occurring non-target species have to be carried out. However, expectations of extensive field testing were not met, as this was a preliminary study on a subset of samples. For qPCR assays targeting a particular species of interest, the assayID program can be used as a preliminary means to identify candidate primer/probes. Further testing with manual *in silico* techniques and software is recommended for all automated selections prior to ordering a costly hydrolysis probe. The optimized assays were shown to be highly effective at detecting target species DNA down to a low concentration as denoted by their LODs (0.0001 ng/µL for sperm whale, porbeagle shark and basking shark, 0.00001 ng/µL for dolphins; Klymus et al. (2020)). Therefore, non-detections of target species throughout the course of this study are likely due to the lack of (or extremely

low concentrations of) quantifiable target DNA in the extract (Eichmiller et al. 2016; Hunter et al. 2017). The LOD of UCC's SYBRgreen assay was one dilution point higher than the LOD of the TaqMan (hydrolysis probe-dependent) assays. We hypothesize that this is due to the efficiency at which target DNA can hybridize to the primer pair, whereas the primer pair + probe assay for TaqMan-based qPCR is highly specific and may not bind to all target DNA within an extract, as suggested by other comparative studies (Cao and Shockey 2012; Zhang et al. 2015).

Detection rates across all qPCRs varied exceptionally depending on the species: sperm whales and dolphins were detected in 43 and 56 qPCR replicates, respectively, whereas shark species were only detected in three (in the case of basking shark) and 13 (porbeagle shark) qPCR replicates and were completely absent from the conservative dataset. This is justified from previous environmental DNA work with shark species, which report low concentrations of eDNA from elasmobranch (sharks and rays) taxa (Dunn et al. 2023). In contrast, extracts generated by all partner laboratories amplified sperm whale DNA across almost all replicates for three separate eDNA samples and dolphin DNA in eight samples for more than one qPCR replicate. Marine mammals notably lose sloughed skin and dispel fecal matter while resting at the surface of the water column in between feeding events (Whitehead et al. 1990; Konrad et al. 2018). Therefore, the genetic material that was collected during sampling events near surfacing individuals likely provided sufficient quantities of eDNA to be collected, filtered and extracted. Not only does the behavior of each species affect their ability to be detected by eDNA, but differences in field sampling may have also factored into detection probability. We attribute the low frequency of detections for IMR's assay (basking shark) in both the all-inclusive and conservative datasets to field sampling at too-great distances (spatially and temporally) from the target species in order to identify significant concentrations of eDNA. Additionally, water volumes of samples taken near sharks (both species) were 5 liters, whereas sperm whale samples were all 10 liters - this variation of water volume is due to the samples being part of a larger field sampling optimization initiative across all eWHALE partners during the sampling season of 2023. For the purpose of the present study, samples were not directly compared to each other. Lysate volumes from INRAE Sylphium filters were higher (up to 6 mL in some instances compared to 1.5 mL from UIBK Sylphium filters) due to improper drying of the filter, which may have consequently diluted the eDNA to a point at which it was no longer detectable. Though only two liters of water were collected near dolphins, their tendency to travel closely in groups at the water's surface likely enhanced the amount of eDNA present (Shane et al. 1986; Acevedo-Gutiérrez and Parker 2000; Gridley et al. 2017). Hence, while the extraction protocols did impact the capability of partner laboratories to detect different species, there are many other factors to account for prior to ecological interpretations of these detections.

There was a significant difference observed between combinations of laboratory-specific extraction and the subsequent target species. This implies that the nature of the sample (e.g. different environmental parameters at the sampling site) could have affected the efficiency of DNA extraction following its collection from the field. Such a theory is consistent with the findings of the Lear et al. (2018) review concerning eDNA extraction, storage, amplification

and sequencing methods. Thus, while particular extraction techniques may perform well with certain sample types (e.g. samples including PCR inhibiting substances), they may not be universally applicable across all eDNA samples, introducing significant discrepancies especially in the context of inter-laboratory comparisons. For example, DNA extracts generated by INRAE yielded lower total DNA measurements than other laboratories for their own eDNA samples (Sylphium filters, target species: porbeagle shark), as well as UIBK eDNA samples (Sylphium and Smith-Root filters, target species: sperm whale), yet similar measurements to other laboratories for UCC samples (target species: dolphin species, Sterivex filters) and IMR samples (target species: basking shark, Sterivex filters). This suggests that the NucleoSpin Tissue Kit may be most effective with DNA lysed from Sterivex filters, which is in accordance with Tsuji et al. (2019) who present that each commercial DNA extraction kit has shown dependence on a combination of the eDNA collection method and physico-chemical parameters of water samples (e.g. the degree of inhibition). Despite these observations, the significantly lower amounts (i.e. higher Ct values) of target DNA throughout INRAE extracts were unexpected for this study. Previous comparisons of extraction performance of NucleoSpin and DNeasy for tissue samples and eDNA samples have shown similar performance (Martincová and Aghová 2020; Myler et al. 2024). Therefore, this finding warrants future exploration by INRAE. However, based on the comparison of their extracts in regards to target DNA detection, this does not immediately necessitate changes to their extraction protocol.

For samples in which inhibition (compounds which may disrupt PCRs) may be expected (e.g. in turbid marine environments), inhibitor-removal kits and/or additional extraction protocol steps are often utilized to enhance the detection of target DNA (Rees et al. 2014). For the purposes of this study, however, all partner laboratories agreed to avoid inhibitor removal steps. Ultimately, the implementation of various Internal Positive Controls corroborated the lack of inhibition across all generated DNA extracts, confirming our choice to not risk the loss of target DNA via an additional inhibition removal protocol. The use of high-quality PCR chemistry could have also influenced the detection success of each qPCR assay (Beng and Corlett 2020; Thalinger et al. 2021), but this effect was not specifically tested in the current study. However, external factors, namely the effect of shipment of eDNA lysate and extracts, were included in preliminary analyses, but did not show any significant effect on the resulting concentrations of DNA per extract.

A total of 36 positive amplifications fell below the assays' LOD and were excluded from one of the two datasets used for statistical testing. Although the results of statistical tests between the full dataset (with all positive amplifications regardless of the assay's LOD) and the subsetted dataset showed the same result - IMR's DNA extracts amplified the target species significantly less than the other lab's extracts - the detection rate of each lab's DNA extracts decreased by approximately 20% (41% in the case of IMR) in the subsetted dataset. The analysis of eDNA detections above or below an assay's LOD is an important factor to consider for future interpretations of eDNA samples in marine ecosystems, in which genomic traces are expected to be far lower than the desired confidence of detection (Paul et al. 1987; Collins et al. 2018). On that note, almost all positive detections which fell above the assay's LOD

were positively sequenced to match the target species' DNA, proving that the above-LOD amplifications were true positives of the target species. However, for the purpose of a cross-laboratory comparison, the dataset provided in the conservative dataset (amplifications at or below the LOD) is most appropriate as stated by Klymus et al. (2020). Ultimately, the DNA was extracted from the same environmental sample and should theoretically be the same across independent laboratories.

Conclusions

Overall, this study demonstrates a comprehensive effort to evaluate the consistency and accuracy of DNA extraction across four different laboratories. While some discrepancies in one laboratory's extraction performance were observed, our findings highlight the overall robustness of eDNA analyses amongst the eWHALE partners. Though three of the four laboratories used similar kits, this analysis demonstrates the variability introduced by subtle modifications to extraction protocols, as well as the critical importance of interpreting data within the context of a given assay's Limit of Detection. As a result of this study, adjustments were made to improve IMR's extraction protocol, thereby enhancing its effectiveness. This research demonstrates the necessity of conducting preliminary validation tests for research projects involving multiple laboratories, ensuring reliable and comparable results. Our work represents a significant step towards the successful implementation of standardized protocols, promoting consistent performance across an international environmental DNA initiative.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

Bottlenose dolphin samples were collected under the NPWS license DER-CETACEAN-2023-67. Porbeagle shark samples were collected under permit number 708/2023 (delivered 6 June 2023). Basking shark eDNA samples were collected onboard "Rind" provided by the Directorate of Fisheries.

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Author contributions

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Data availability

All data used for analysis in this study are available in Suppl. materials 8, 10, and 11.

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Supplementary material 1

Extraction protocol of eDNA lysates at UIBK using the Qiagen BioSprint® 96 Workstation

Authors: Bettina Thalinger

Data type: pdf

Explanation note: This file provides a step-by-step protococl for preparing samples and reagants for their use in the Qiagen BioSprint® 96 Workstation for DNA extraction. This information is also published here: https://dx.doi.org/10.17504/protocols.io.q26g71p83gwz/v1.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl1

Supplementary material 2

qPCR optimization protocols per assay

Authors: Lauren Rodriguez, Teddy Urvois, Lorenzo De Bonis, James McKenna

Data type: pdf

Explanation note: This file outlines the optimization for each assay used in this study for the four target marine megafauna species. Steps of optimization include gradient qPCRs to optimize the annealing/extension temperature as well as testing the assay with multiple nontarget species tissue.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl2

Supplementary material 3

End-point PCR protocol followed by INRAE for sequencing preparation

Authors: Teddy Urvois

Data type: pdf

Explanation note: This file outlines the PCR chemistry and conditions implemented by INRAE for the amplification of porbeagle shark DNA from extracts used in this study to be sent for Sanger sequencing.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl3

Supplementary material 4

Level of validation scale (as presented by Thalinger et al. 2021) for each qPCR assay

Authors: Lauren Rodriguez, Bettina Thalinger

Data type: pdf

Explanation note: This file presents the levels at which each assay (sperm whale, porbeagle shark, dolphin species, and basking shark) meets the criteria of Thalinger et al. 2021, who presented a 5-level validation scale that should be met for species-specific assays.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl4

Supplementary material 5

Positive qPCR amplifications of each species per lab

Authors: Lauren Rodriguez, Teddy Urvois, Lorenzo De Bonis, Jack McKee, James McKenna

Data type: pdf

Explanation note: This file presents a detailed summary of the target species which were (or were not) amplified within each participating laboratory's DNA extracts. The information is provided for both datasets (all-inclusive and conservative, based on the assay's Limit of Detection) used in this study.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl5

Supplementary material 6

Triplicate Qubit Measurements

Authors: Lauren Rodriguez, Teddy Urvois, Lorenzo De Bonis, Jack McKee, James McKenna

Data type: pdf

Explanation note: This figure represents an expanded version of Fig. 2 from the text, in which Qubit fluorometry measurements are presented for each laboratory's extracts for all samples used in this study. Each plot is labeled by the source in which the samples originated (i.e., different target species).

Link: https://doi.org/10.3897/mbmg.9.128235.suppl6

Supplementary material 7

Ct Values for Ring Test Extracts

Authors: Lauren Rodriguez, Teddy Urvois, Lorenzo De Bonis, Jack McKee, James McKenna

Data type: pdf

Explanation note: This figure presents Ct values for each ring test extract (in triplicate) with points colored by the participating laboratory. Panel A) represents the all-inclusive dataset whereas panel B) represents the conservative dataset (detections at or below the respective assay's Limit of Detection). The information in this figure is a different representation of the same data presented in Fig. 4A, B.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl7

Supplementary material 8

Metadata of eDNA samples

Authors: Lauren Rodriguez, Lorenzo De Bonis, Caterina Lanfredi, Jack McKee, James McKenna, Teddy Urvois

Data type: xlsx

Explanation note: This file provides the sampling parameters (target species, water volume, latitude/longitude, filter type) of eDNA filters that are analyzed in this study. This file also provides information on the lysate and DNA extract volumes, incubation time, and presence of Internal Positive Controls for each sample.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl8

Supplementary material 9

Species-specific assay descriptions

Authors: Lauren Rodriguez, Teddy Urvois, Lorenzo De Bonis, James McKenna

Data type: xlsx

Explanation note: This file, adapted from Klymus et al. 2020, provides a detailed description of each of the four DNA assays implemented in this study. Three assays are unique and published as a part of this study whereas one assay was modified from a previously-existing primer set.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl9

Supplementary material 10

Qubit Data Measurements

Authors: Lauren Rodriguez, Teddy Urvois, Lorenzo De Bonis, Jack McKee, James McKenna

Data type: csv

Explanation note: This file provides the readings of total DNA (ng.µl) from Qubit fluorometry triplicate measurements of each eDNA extract used in this study.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl10

Supplementary material 11

qPCR Data Measurements

Authors: Lauren Rodriguez, Teddy Urvois, Lorenzo De Bonis, Jack McKee, James McKenna

Data type: csv

Explanation note: This file provides Cycle number (Ct) values and detections (yes/no) for each eDNA extract (in triplicate measurements) used in this study.

Link: https://doi.org/10.3897/mbmg.9.128235.suppl11